Molecular Cloning of Chalcone Isomerase Genes from Soybean and Its Application in Color Engineering

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ABSTRACT

Flower color is an important trait of ornamental flowers. Some beautiful flower crops and varieties are lack of specific colors. For this purpose, the collaborative proposal includes pigment gene cloning, transformation vector construction, gene transformation, and mass propagation of transgenic plants in order to develop new flower (or leaf) colors for chrysanthemum, caladium and anthurium. Three full length chalcone isomerase (CHI) cDNAs have been cloned from the black seed coats of soybean among them CHI1 belongs to type II and CHI2 and CHI3 belong to Type I, respectively. These three CHI genes may with different function and role in the synthesis of isoflavanone and anthocyanins. Theoretically, the yellow flower can be achieved by accumulation of yellow narigenin chalcone in the flavonoid biosynthetic pathway by inhibition of endogenous CHI gene in flower through anti-sense or RNAi technology. For this purpose, the CHI cDNAs were constructed into the pCAMBIA series vectors driven by a 35S promoter in either sense or anti-sense for agrobacterium-mediated transformation to ornamental crops such as, chrysanthemum, caladium and anthurium to obtain yellow color which are not commercially available in flower market. The CHI1 gene has been successfully transferred into chrysanthemum in either sense or anti-sense direction. Flower color of transgenic caladium Red Star with sense CHI construct turns from red to green indicating that the overexpressed soybean CHI gene suppresses the endogeneous CHI gene expression and blocked ntocyanin accumulation on leaves. This result provides an alternative way to engineer color through transformation of the CHI gene. As we know, this is the first report on color engineering in ornamental plants by transformation of CHI gene.

Key words: Soybean, Chalcone isomerase, Color, Caladium, Chrysanthemum, Transformation.

INTRODUCTION

There are always desires for diversities of flower colors from the point of view of consumer therefore who can create new varieties in a short time would have strong competent in market (Mol *et al.*, 1995). From all prospects, the genetic transformation will fit this acquirement properly (Tanaka *et al.*, 1998). Flower colors

are decided by many factors and among them pigments of anthocyanin is the major group (Inagaki *et al.*, 1999). Anthocyanin pathway is one of unique and well-studied pathway of plant however most studies were focused in few model plants like maize, snapdragon and petunia (Mol *et al.*, 1989; Oud *et al.*, 1995; van Houwelingen *et al.*, 1998). Among all genes in anthocyanin pathway, DFR (dihydroflavonol reductase) gene has played an important role for converting dark colors like orange, brick red and purple (Tanaka *et al.*, 1998; Mol *et al.*, 1989). It is thought that by transferring proper DFR gene(s) into the plants lack of key enzyme would accomplish in the very end of anthocyanin pathway to make dark colors theoretically. In the past few years in our laboratory we cloned seven groups of pigment genes including: 4CL, CHS, CHI, F3H, F3'H, DFR, F3'5'H, and GST genes from a black seed coat soybean 'Chin-Ren-Woo-Dow' (CRWD).

Biolistic particle bombarment and *Agrobacterium*-mediated transformation are the most popular two methods used for plants and both methods have many successful examples in various species (Kikkert, 1993; Morrish *et al.*, 1993; Hooykaas and Schilperoort, 1992; Sanford *et al.*, 1993; Smith and Hood, 1995) . In this study, the *Agrobacterium*-mediated transformation method was applied to pigment gene transformation for engineering new colors for both flower and ornamental leaf crops such as chrysanthemum and caladium.

MATERIALS AND METHODS

Plant materials

Seed coats of soybean were harvested from developing seeds of CRWD variety with the *iRT* genotype. Total RNA was isolated by BSA-PVPP-PK method (Wang and Vodkin, 1994) for RT-PCR, northern hybridization and construction of cDNA library. Most molecular techniques were followed the protocols of Sambrook *et al.* (1989) if there is not specified.

Construction of a CRWD cDNA library

The poly(A)$^+$ RNA was prepared from the total RNA using a Dynabeads mRNA Purification Kit (Dynal-Product No.610.01). The cDNA was prepared using 5μg of poly(A)$^+$ RNA according to the instructions for a cDNA Synthesis Kit, ZAP-cDNA Synthesis Kit, and ZAP-cDNA Gigapack III Gold Cloning Kit (Stratagene). The first-strand cDNA was synthesized using MMLV-RT. The second-strand synthesis, *Escherichia coli* ribonuclease H and *E. coli* DNA polymerase I were used. The cDNA was ligated to *Eco*RI adaptors, and then cloned into the *Eco*RI site of Uni-ZAP XR vector, packaged and plated on selective XL1-Blue MRF' cells. The result library contained ca. 1×10^6 p.f.u. independent clones.

Genomic library construction and screening for CHI gene

The soybean genomic library was constructed using genomic DNA of CRWD (*iRT* genotype) variety (C. S. Wang *et al.* unpublished) for screening *CHI* gene.

Molecular cloning of CHI2 and CHI3 genes by genome walking

Genome walking technique developed by BD Biosciences (GenomeWalker Kit) was applied for cloning the flanking sequences of CHI genes from soybean genome. A primer, 5'-TGCTCGACTCCCTTGACTTCTACAG-3', was designed for the first round of genome walking. Two DNA fragments were amplified from *Dra* I and *Pvu* II libraries

constructed from restriction enzyme digested genomic DNA of UC9 (*iRT* genotype). To amplify the entire *CHI2* gene, a new primer, 5′-TTGCATGCCTTGA AGATCTGATAAC-3′, was designed for second round of genome walking. A specific DNA fragment (3.0 kb) amplified from *Eco*R V library was cloned and sequenced to complete the *CHI2* genomic sequences. A partial 3′ end gene sequences of the third CHI gene, *CHI3*, was found in the opposite direction by BLAST analysis. The amino acid sequences *CHI1* and *CHI2* are around 70% identical to the Type II CHI of leguminous plants and show low identity to non-leguminous plants (Table 1 and 2). Type I CHI only recognizes 6′-hydroxylasechalcone as a substrate, however, type II CHI catalyzes both 6′-hydroxychalcone and 6′-deoxychalcone.

Table 1. The comparison of amino acids sequence identity of soybean CHI1 with the same proteins published in Genbank.

Species	Identity (%)	Accession No.
Legume		
Pueraria lobata	87	BAA09795
Phaseolus vulgaris	82	CAA78763
Medicago sativa	77	AAB41524
Lotus japonicus CHI3	71	BAC54038
Lotus japonicus CHI1	69	BAC53983
Lotus japonicus CHI4	71	CAD69022
Pisum sativum	68	AAA50174
Non-legume		
Dianthus caryophyllus	53	CAA91931
Petunia hybrida CHIB	53	CAA32730
Ipomoea batalas	52	BAB58538
Ipomoea purpurea	53	AAB86474
Elaeagnus umbellate	53	AAC16013
Lotus japonicus CHI2	51	BAC53984
Vitis vinifera	52	CAA53577
Saussurea medusa	51	AAM48130
Citrus sinensis	52	BAA36552
Allium cepa	48	AAU11844
Petunia hybrida CHIA	50	CAA68769
Callistephus chinensis	50	CAA91921
Eustoma grandiflorum	49	BAD34458
Oryza sativa	4	AAO65886
Raphanus sativa	48	AAB87071
Arabidopsis thaliana	48	CAB94968
Zea mays	48	CAA80441
Hordeum vulgare	46	AAM13449

Table 2. The comparison of amino acids sequence identity of soybean CHI2 with the same proteins published in Genbank.

Species	Identity (%)	Accession No
Legume		
Phaseolus vulgaris	68	CAA78763
Pueraria lobata	69	BAA09795
Glycine max CHI1	69	AAK69432
Medicago sativa	66	AAB41524
Lotus japonicus CHI3	67	BAC54038
Lotus japonicus CHI1	67	BAC53983
Lotus japonicus CHI4	68	CAD69022
Non-legume		
Ipomoea batalas	56	BAB85838
Elaeagnus umbellate	57	AAB86474
Ipomoea purpurea	55	AAC16013
Vitis vinifera	53	CAA53577
Saussurea medusa	51	AAM48130
Lotus japonicus CHI2	56	BAC53984
Petunia hybrida CHIB	54	CAA32730
Callistephus chinensis	52	CAA91921
Dianthus caryophyllus	53	CAA91931
Petunia hybrida CHIA	50	CAA68769
Citrus sinensis	55	BAA36552
Allium cepa	47	AAU11844
Eustoma grandiflorum	52	BAD34458
Pisum sativum	57	AAA50174
Arabidopsis thaliana	50	CAB94968
Raphanus sativa	49	AAB87071
Zea mays	51	CAA80441
Hordeum vulgare	46	AAM13449
Oryza sativa	47	AAO65886

Pigment gene construction, transformation and transgenic plant screening

The cDNA sequence of CHI has been inserted into pCambia vector series for agrobacterium-mediated transformation. Both sense and antisense vectors are constructed for transformation purpose. The sense pLMGDFR1GUS and anti-sense pLMGDFR1AGUS plasmid consisting of a *uidA* reporter gene coding for β-glucuronidase (GUS) and a hygromycin phosphotransferase (*hpt*) selectable marker gene was used for transformation. Both genes are controlled by the cauliflower mosaic virus (CaMV) 35S promoter and followed by nopaline synthatase (NOS) polyadenylation sequences. Binary vectors of sense pLMGDFR1GUS and anti-sense pLMGDFR1AGUS plasmid in *Agrobacterium*-mediated transformation system will also be

tested in this project for these two flower crops. Genomic DNA was extracted from each regenerants screened from the antibiotic-containing medium. PCR and Southern hybridization were applied to screen and/or confirmed the transgenic plants. The putative transgenic plants were carefully planted in the greenhouse for flower color investigation and pigment analysis.

Callus induction and shoot regeneration of anthurium and caladium

The " Feugo" variety of *Anthurium andraeanum* and the " Freida Hemple" variety of caladium was selected as experimental materials. Comparison on the effect of medium formulation and illumination on the induction and regenerating of callus and shoot from the petiole and leaf disk of tissue cultured plantlets. Culture medium: A. Callus induction medium: T0-t：1/8 MS、BA 1ppm、2,4-D 0.08ppm; T0-p：1/2 MS、TDZ 0.5ppm, and B. Shoot regeneration medium: T1-t：1/8 MS、BA 1ppm and T1-p：1/2 MS、BA 0.1ppm. Illumination treatments: light (L, 16/8hr) and dark (D) treatments. Experiments were conducted in combination of medium and illumination treatments.

Regeneration and pigment gene transformation in chrysanthemum and caladium

Plasmids of pigment genes, CHI, DFR constructed in pCambia series vector with various selection markers (cefatoxime, hygromycin), reporter genes (GFP, GUS) in either sense or anti- sense direction were used for transformation. Agrobacterium, EHA105 or LBA4404 strain, transformed with plasmid of pigment gene was prepared according to Sherman et al. (1998) with modifications. Explants (stem segment or leaf) pre-cultured for five days were used for transformation. Explants were dipped with freshly prepared agrobacterium (OD_{600}=0.6) were cultured in regeneration medium (MS medium with 1mg/L BA, 0.1 mg/L NAA) with 100μM acetosyringone at 22℃ for two days in the dark. After three times washing with sterilized water, the explants were washed with 500ppm Cefatoxime then cultured in the regeneration medium (containing 500ppm Cefatoxime) in the dark for four days. Transformed plants were screened in the selection medium containing Cefatoxime 500ppm and hygromycin 15ppm and cultured at 25℃ in the light and sub-cultured every two weeks. Green shoot emerged from the wounded region of explant could be observed at about 60 days after culture.

Analysis of flower pigments by HPLC

Petals of plant materials were harvested and anthocyanin pigments were extracted with 1% HCl-MeOH followed with 1.5 N HCl and quantified by spectrophotometry at A_{530} then, calculated by C=A/38000 L/mole×1.2 cm /molar. The HPLC qualification was conducted at mobile phase：0.425% phosphoric acid：MeOH = 1：1；wave length：530 nm；flow rate：1 ml/min （0.425% phosphoric acid：MeOH = 1：1） using a Inertsil ODS-3 column（5 μm, 4.6×250 mm, GL Sciences Co., Japan）in a Waters HPLC (USA) as described by Reddy et al. (1995).

RESULTS AND DISCUSSION

Three CHI cDNAs were cloned from soybean

Three CHI cDNAs, CHI1, CHI2, and CHI3, were screened from the cDNA library made from young (<100mg) seed coats of CRWD variety (Fig.1, 2), however, the Southern analysis (data not shown) showed that only one copy of CHI gene was

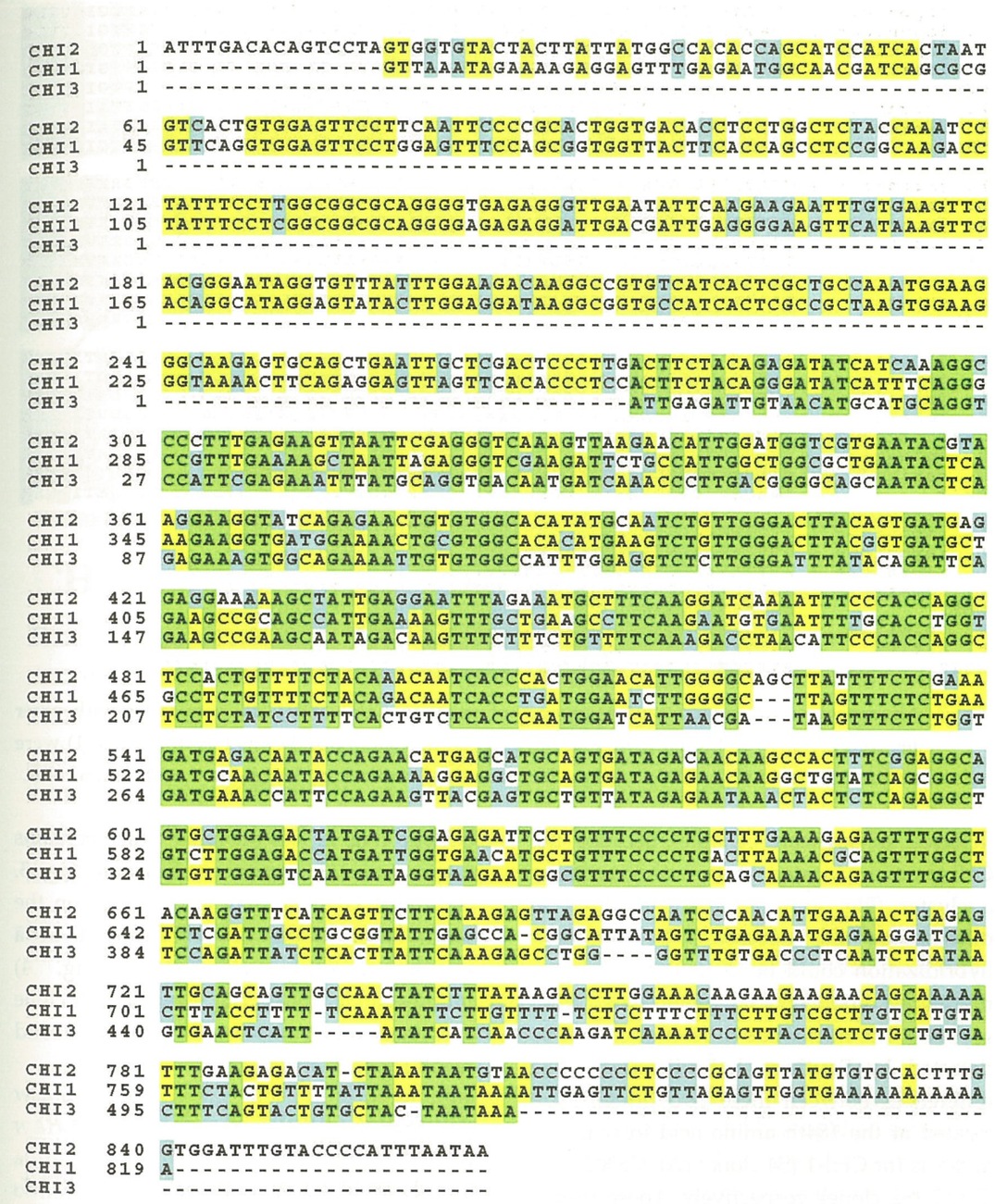

Fig. 1. Alignment of three soybean *CHI* gene sequences. Complete sequences of soybean CHI1, CHI2 and partial sequences (3′ end) of CHI3 are aligned using CLUSTALW program. The conserved regions are highlighted with green color.

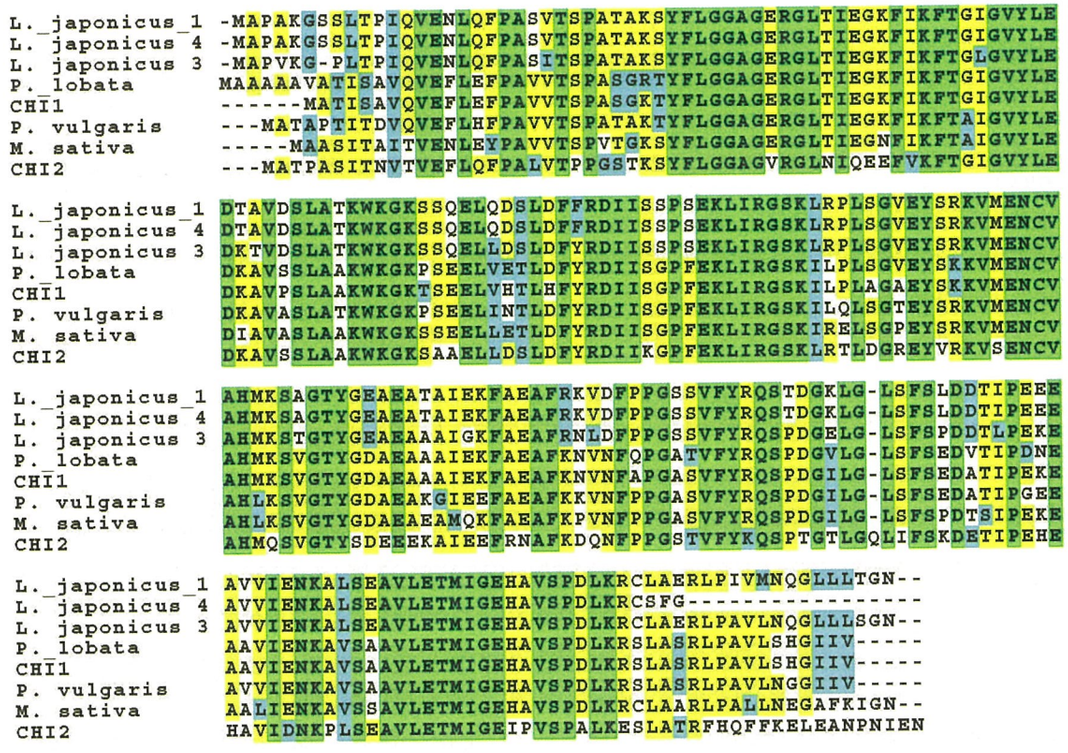

Fig. 2. Alignment of the amino acid sequences of soybean *CHI1* and *CHI2* sequences with other legume *CHI*s. Type II CHI from legume species (accession number as listed in Table 1) were aligned using CLUSTALW program. The conserved regions are highlighted with green color.

detected in soybean genome. Because the CHI genes are located on soybean genome as a cluster (Fig. 4) it is reasonable that to differentiate the difference by Southern hybridization could be difficult. This is the first paper report on CHI genes cloned from soybean. The nucleotide difference cannot be detected by Southern hybridization. The differences between these two proteins are located at the 184th amino acid in which A and T is for CHI-1 (S4 clone) (AF276302) and CHI-2 (S2 clone), respectively. These results suggested that the CHI gene in soybean genome may exit as a duplicate gene therefore the cloning of CHI gene will answer the question. By genomic library screening, a genomic clone, *CHI* 1, with 8,520bp (Fig. 2) was obtained which contains 4 exons and 3 introns as shown in Fig. 3. Another CHI gene may also located on the same chromosome of soybean but in a reverse direction as *CHI* 1 (Fig. 4) unfortunately, this is not a full length clone and need to be completed to provide a solid result for gene structure.

Molecular cloning of CHI2 genes by genome walking

A technique, genome walking, developed by BD Biosciences and the GenomeWalker Kit, was applied for cloning the flanking sequences of CHI genes from soybean genome. A primer, 5′-TGCTCGACTCCCTTGACTTCTACAG-3′,

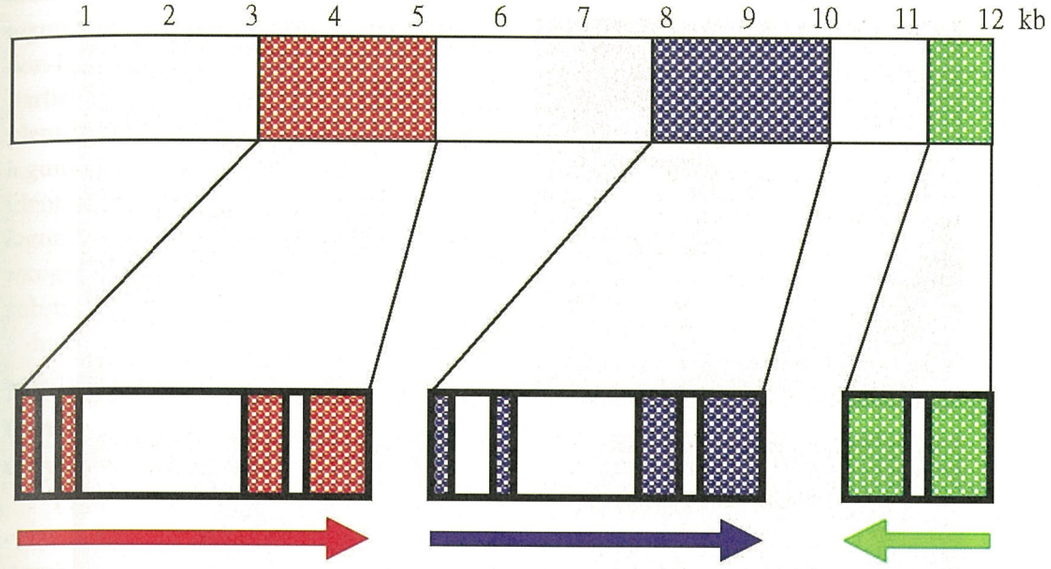

Fig. 3. Gene structures of soybean *CHI* genes. Three colored blocks represent different *CHI* genes of soybean. The red , blue and green boxes represent for the *CHI1*, *CHI2* and *CHI3*, respectively. Colored boxes represent exons while the empty boxes represent for introns. The arrow represents direction of transcription of each *CHI*.

was designed for the first round of genome walking. Two DNA fragments were amplified from *Dra* I and *Pvu* II libraries constructed from restriction enzyme digested genomic DNA of UC9 (*iRT* genotype). To amplify the entire *CHI2* gene, a new primer, 5′-TTGCATGCCTTG-AAGATCTGATAAC-3′, was designed for second round of genome walking. A specific DNA. fragment (3.0 kb) amplified from *Eco*R V library was cloned and sequenced to complete the *CHI2* genomic sequences. A partial 3′ end gene sequences of the third CHI gene, *CHI3*, was found in the opposite direction by BLAST analysis (Fig. 5). The amino acid sequences *CHI1* and *CHI2* are

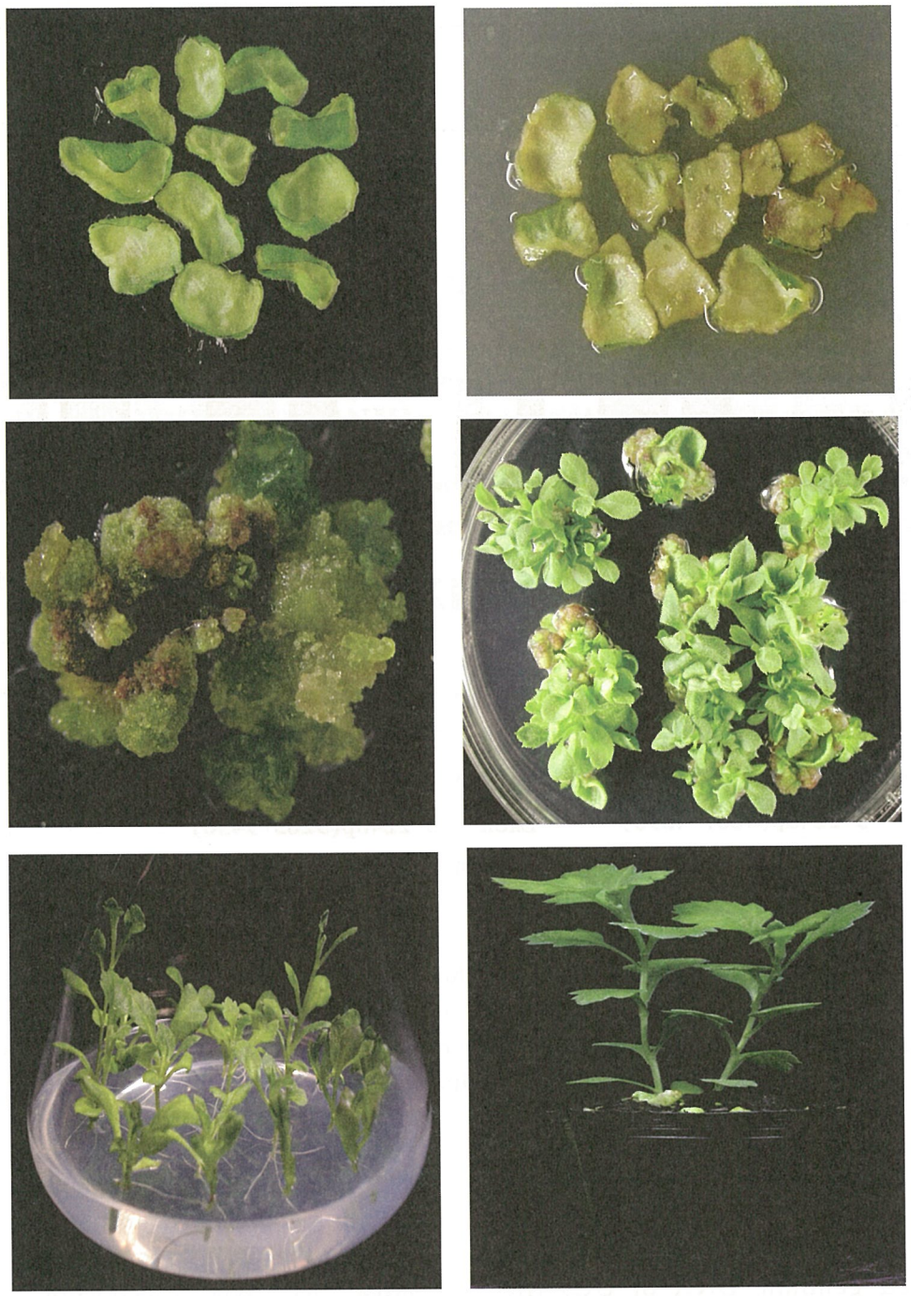

Fig. 4. Flow chart of transgenic chrysanthemum.(A)pre-culture of explant;(B)co-cultivation with agrobacterium;(C)callus induction and regeneration on hygromycin medium;(D)shooting on hygromycin regeneration medium;(E) rooting of regeneration shots on hygromycin selection medium;(F) transgenic plants at six months after transformation.

around 70% identical to the Type II CHI of leguminous plants and have low identity to non-leguminous plants (Table 1 and 2). The partial cDNA sequences of *CHI3* show high identity (about 70%) to type I CHI of non-leguminous plants and with only 50% identities while compared to type II CHI of leguminous plants Type I CHI only recognizes 6'-hydroxylasechalcone as a substrate, however, type II CHI catalyzes both 6'-hydroxychalcone and 6'-deoxychalcone.

New flower color developed by transformation of soybean CHI gene to chrysanthemum

Pigment genes were transferred to chrysanthemum and caladium by *Agrobacterium*-mediated method, putative transgenic plants were screened in the selection media containing proper antibiotics and followed with PCR to obtain transgenic plants with CHI gene. An efficient transformation system has been established in this proposal as demonstrated in the Fig. 5. Though there exits cultivar specific problems in the transformation of chrysanthemum, our system can be modified for many commercial varieties. With this system, the transformation process can be completedwithin nine months to obtain flowering plants in crysanthemum. We have obtained 28 different transgenic anti-sense CHI chrysanthemum plants (Fig. 4). After antibiotic screening, they were further screened for both GUS and CHI genes by PCR. The flowers colors were expected to be yellow if the expression of CHI gene was inhibited by transgene however, the flowers of transgenic mum show only light yellow at the early stage of flower development (Fig. 4). The explanations for this observation is that: 1.there may not have narigenin chalcone accumulated in the transgenic plants because of lack of CHS in this white color variety, 2. the soybean CHI gene may not be able to complete block the expression of CHI gene in chrysanthemum due to the less similarity in DNA sequences. However, further experiment is required to answer this observation.

Interesting, several transgenic plants (Fig. 5) with the over-expression CHI construct show altered color from red control to yellow suggesting that the expression of endogenous CHI gene is suppressed by the transformed soybean CHI gene as reported by Meyer et al., (1987) when transferred the CHS gene of maize to petunia. According to HPLC analysis, the original Red Beauty flowers contains only cyaniding and show red color, however, the transgenic CHI plant contains lutein, β -carotene of carotenoid biosynthesis pathway but not cyanidin and show yellow color suggesting that the endogenous anthocyanin biosynthesis pathway has been blocked.

As we know, our experiment provides the first example of flower color engineering through transformation of a heterogeneous CHI gene from soybean. Besides, we also demonstrate that plant metabolic pathway could be altered through transformation of a similar gene differs in gene function.

Similarly, soybean CHI1 genes were also transferred to caladium by *Agrobacterium*-mediated method, putative transgenic plants were screened in the selection media containing proper antibiotics as previously described or can be subjected to fluorescent microscope detection for selection marker 'GFP' expression. An agrobacterium-mediated transformation system has established for caladium with high efficiency. Transgenic plants with anti-sense CHI gene show three types of leaf colors as compare

with control. The first type of transgenic caladium show similar color as control plants. The second type shows very light red on their leaves, the third type of transgenic plants shows almost no red pigment accumulated on their leaves (Fig. 6). The pigment biosynthesis on the leaves of the type II and type III were inhibited by various levels of anti-sense CHI gene of soybean

Wild type Transgenic CHI Wild type Transgenic CHI

Fig. 5. Flowers of transgenic CHI chrysanthemum show yellow color that accumulates lutein and carotene instead the red cyanidin of the wild type (Red Beauty).

Fig. 6. The leaves color of control (upper most) and transgenic anti-sense CHI caladium plants.

and show no red pigment accumulated, no yellow color was observed on leaves as we have expected. A reasonable explanation is that the yellow color of narigenin chalcone was covered by green chlorophyll therefore, yellow color cannot be visible. Biochemical analysis will provide reasonable explanation for this observation. In the future, proper variety will be chosen for pigment transformation to obtain yellow or blue-purple caladium. As we know, this is the first report on leaf color engineering on caladium and ornamental leaf plants. Because, all the mass propagation systems for both chrysanthemum and caladium have well established, any good transgenic plants can be commercialized within one year.

ACKNOWLEDGEMENTS

The financial support on project from COA to C. S. Wang and on travel to the USA code number: 93-SA-1.4-IC-01(1) of American Institute at Taiwan (AIT) to C. S. Wang and Peter, H. J. Shen are acknowledged.

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